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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Mercy M. Davidson
Serial No. : 09/604,876 Examiner: R. Schnizer
Filed : June 28, 2000 Group Art Unit: 1635
For : IMMORTALIZATION OF HUMAN POST-MITOTIC CELLS

1185 Avenue of the Americas
New York, New York 10036

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. § 1.132

I, Mercy M. Davidson, Ph.D., hereby declare that:

1. I am the inventor named in the above-identified patent application.
2. I am also presently a Research Scientist in the Department of Neurology at Columbia University in New York, NY. A copy of my curriculum vitae is attached hereto as **EXHIBIT A**.
3. I have reviewed and am familiar with the subject application, including pending claims 1 and 3-5. I understand that pending claims 1 and 3-5 provide an immortalized human undifferentiated cardiomyocyte cell line wherein the cell line is produced by a method comprising the step of fusing a post-mitotic primary non-immortalized human cardiomyocyte with a human fibroblast, the fibroblast (a) having been treated with ethidium

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bromide; (b) comprising a replicable vector expressing SV40 large T antigen which confers immortality on a cell comprising same; and (c) being free of mitochondrial DNA.

4. I have read and am familiar with the January 27, 2004 Office Action issued by the United States Patent and Trademark Office in connection with the subject application. I understand that in the January 27, 2004 Office Action, the Examiner rejected claims 1 and 3-5 under 35 U.S.C. §102(b) as allegedly anticipated by Wang et al., "Establishment Of A Human Fetal Cardiac Myocyte Cell Line", In Vitro Cell. Dev. Biol., 27A:63-74, January 1991 (hereinafter "Wang"). A copy of Wang is annexed hereto as **EXHIBIT B**.
5. Specifically, I understand the rejection under 35 U.S.C. §102(b) to be based on the Examiner's assertion that the human fetal cardiac myocyte cell line taught by Wang, i.e., the "W1" cell line, is the same as the cell line of claims 1 and 3-5 (i.e., the "claimed cell line").
6. I have read and am familiar with Wang and its teachings, in particular that of the W1 cell line.
7. The claimed cell line is not the same as the W1 cell line of Wang for the reasons set forth below.
8. Images of the claimed cell line are shown in **EXHIBIT C**. Specifically, **EXHIBIT C** sets forth two

light microscopic images of cells from a transformed human adult cardiomyocyte cell line designated "AC16" (panel (a)) and a transformed human fetal cardiomyocyte cell line designated "RL14" (panel (b)). Both the AC16 and RL14 cell lines are immortalized human undifferentiated cardiomyocyte cell lines. The AC16 cells were generated by fusing post-mitotic primary non-immortalized human cardiomyocytes obtained from primary adult human ventricular tissue with ethidium bromide-treated, human SV-40-transformed mitochondrial DNA-free (rho zero) fibroblasts. The RL14 cells were generated by fusing post-mitotic primary non-immortalized human fetal cardiomyocytes with the same ethidium bromide-treated, human SV-40-transformed mitochondrial DNA-free (rho zero) fibroblasts used to generate the AC16 cells. The only difference between the AC16 and RL14 cell line production methods was the use of primary cardiomyocytes at developmentally different stages (i.e., adult or fetal). Adult stage primary cardiomyocytes were used to generate the AC16 cell line, and fetal stage primary cardiomyocytes were used to generate the RL14 cell line.

9. The claimed cell line, whether derived from adult or fetal primary cardiomyocytes, possesses certain morphological characteristics. As shown in **EXHIBIT C**, the AC16 and RL14 cell lines are characterized by homogeneous cells which (a) are evenly-shaped, (b) have large central nuclei and

(c) have a larger average ratio of nucleus volume to cytoplasm volume than do native primary human cardiomyocyte cells. Both AC16 cells and RL14 cells are also smaller in size relative to native primary human cardiomyocytes.

10. Figures 1 and 5 of Wang show the W1 cell line. In contrast to the claimed cell line, the W1 cell line shown in Figures 1 and 5 of Wang contains heterogeneous, refractile, spindle-shaped cells. The cells of the W1 cell line morphologically resemble native human primary cardiomyocytes. Wang states that the W1 cell line "has been shown to share morphologic and phenotypic characteristics with native human fetal cardiomyocytes," that W1 cells "look very similar" to native primary cardiomyocytes under light and electron microscopy, and that "by morphologic criteria these two types of cells [W1 cells and human fetal cardiac myocytes] are indistinguishable from one another." Wang, page 73, column 1, ¶1; page 66, column 1, 2nd full paragraph; and page 66, legend for Figure 1, respectively.
11. A comparison of morphologies of the claimed cell line (described in paragraphs 8 and 9) and the W1 cell line of Wang (described in paragraph 10 above) reveals that these two cell lines are morphologically different, and therefore, not the same.

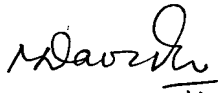
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12. The claimed cell line and the W1 cell line of Wang also have different growth characteristics. **EXHIBIT D** sets forth growth curve data obtained from an experiment using AC16 cells. In this experiment, 5×10^4 cells of each of the three cell types (AC16, control fibroblasts and DWFb1p⁰ (human SV-40 transformed mitochondrial DNA-free [rho zero] fibroblasts)) were seeded in 10 ml of growth medium in multiple 10 mm² dishes. For AC16 cells, DMEM/F12 supplemented with 12.5% FBS was used, and for control fibroblasts, MEM supplemented with 15% FBS was used. To support the growth of DWFb1p⁰ cells, medium was used which consisted of the fibroblast medium and uridine at 50µg/ml. At 24-hour time intervals, cells from individual plates were trypsinized and counted. AC16 cells continued to divide until the 6th day. Proliferation of AC16 cells slowed as the cells approached confluence, similar to the observed proliferation of control primary fibroblasts. The calculated doubling time was 24.49 hours for the AC16 cells, 23.91 hours for the control fibroblasts and 23.09 hours for the DWFb1p⁰ cells.
13. Wang states on page 66, column 2, 1st full paragraph, that the W1 cell line has a doubling time of 55.4 hours. This doubling time is more than twice as long as the 24.49 hour doubling time of the claimed cell line.

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14. Based on the morphological and doubling time differences described above, the claimed cell line is not the same as the W1 cell line of Wang.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made herein on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing thereon.



Mercy M. Davidson, Ph.D.

5. 18. 04

Date

CURRICULUM VITAE

Mercy Mascreen Davidson, Ph.D.
February 24th, 2004

Personal Data

Name: Mercy Mascreen Davidson, Ph.D.
Birthdate: April 7, 1949.
Birthplace: Madras, India.
Marital status: Married, two children.
Citizenship: USA

Academic training

Ph.D. 1979, Biochemistry, Madras Medical College, Madras, India.

Thesis: *Biochemical aspects of neuromuscular diseases.*

Sponsor: K. Valmikinathan, Ph.D., Professor of Neurochemistry, Madras Medical College, India.

M.Sc. 1970, Biochemistry, University of Madras, India.

B.Sc. 1968, Major in Chemistry, Minor in Physics and Biology, Queen Mary's College, Madras, India.

Professional experience

1997- present Research Scientist, and Director of the Laboratory of Tissue Culture, H. Houston Merritt Research Center, Department of Neurology, College of Physicians & Surgeons, Columbia University, New York.

1987- 1997: Associate Research Scientist, Department of Neurology, College of Physicians & Surgeons, Columbia University, New York.

1984-1987: Postdoctoral Research Scientist, Department of Neurology, College of Physicians & Surgeons, Columbia University, New York.

1981-1984: Postdoctoral Research Fellow of the Muscular Dystrophy Association, Department of Pediatrics, College of Physicians & Surgeons, Columbia University, New York.

1977-1981: Lecturer in Neurochemistry, National Institute of Mental Health & Neurosciences, Bangalore, India.

1974-1977: Junior Research Fellow, University Grants Commission, Institute of Neurology, Madras India.

1972-1974: Junior Research Fellow, Indian Council of Medical Research, Institute of Neurology, Madras India.

Honors:

1968: B.Sc. Chemistry with Honors.

1970: M.Sc. Biochemistry with Honors.

1972: Recipient of the Indian Council of Medical Research (ICMR) Merit Scholarship.

1974: Recipient of the University Grants Commission (UGC) Merit Scholarship.
 1981: Recipient of Muscular Dystrophy Association, USA, Jerry Lewis Postdoctoral Fellowship.

Memberships:

American Society for Cell Biology
 American Society for Human Genetics
 American Medical Writer's Association

Fellowship and grant support:

<u>Agency</u>	<u>Grant period</u>	<u>Role</u>
<u>Past</u>		
MDA	5-15-81 to 5-14-83	Postdoctoral fellow
Title of project: <i>Studies of Human Muscle Phosphofructokinase.</i>		
MDA	1-1-94 to 12-31-96	Principal Investigator
Title of project: <i>Kearne-Sayre Syndrome: Studies in Innervated muscle culture.</i>		
NIH	2-1-95 to 11-30-1999	Principal Investigator/Subproject
Title of project: <i>Studies of MERRF and MELAS in nerve-muscle co-culture.</i>		
NIH	12-1-96 to 11-30-2001	Co-Investigator
Title of project: <i>Analysis of mtDNA rearrangements in post mitotic cells.</i>		
AHA	7-1-97 to 6-30-2000	Principal Investigator
Title of project: <i>Analysis of pathogenic mtDNA mutations in human cardiomyopathies.</i>		
<u>Current</u>		
NIH	12-15-99 to 11-30-04	Principal Investigator/Subproject
Title of project: <i>Pathogenesis of MERRF and MELAS in transmtochondrial muscle cultures.</i>		
NIH	2-1-03 to 12-31-07	Co-Investigator
Title of project: <i>Nuclear gene involvement in cytochrome c oxidase deficiency.</i>		
NIH	5-1-02 to 4-30-07	
Title of project: <i>The effect of asbestos on mitochondrial function.</i>		

Post doctoral fellows trained

Claudia Nesti, Ph. D. Sept 2002 to Jan 2004
Studies of the A4300G mutation in human cardiomyocyte cell line.

Carla Giordano, MD July 1998 to March 2001
Cybrid analysis of the A1555T and the A4300G mutation.

Leonardo Salviati, MD, Italy
Copper supplementation in myoblasts from patients with the SCO2 mutation.

Sabrina Sacconi, MD, Italy
Analysis of a novel dominant mtDNA mutation G5545A in fibroblasts and cybrids.

Angela Richardson, Fall semester 2001. Independent Research, Department of Biological Sciences, Columbia University, NY
The effect of dichloroacetate on lactate levels in cybrids harboring a mitochondrial DNA mutation associated with myoclonic epilepsy with ragged red fibers (MERRF).

Claudia Sobreira, MD, Sao Paulo, Brazil, April 1993 to 1996
Studies of mtDNA complementation of non-overlapping deletions in post-mitotic cells.

Uli Walker, MD, Freiburg, Germany, 1993 to 1995
Studies of mtDNA complementation of the MELAS and MERRF point mutations in post-mitotic cells

Teaching experience:

1977 - 1981 - Courses taught to students of D.M.(Neurology), and D.P.M. (Psychiatry), National Institute of Mental Health & Neurosciences, Bangalore, India.
I semester - Biochemistry of neurological disorders.
II semester - Biochemistry of neuromuscular diseases.

Lectures:

“Maternal inheritance and mutations in the mitochondrial genome”. 1995 - Graduate program research lecture, Department of Biology, Farleigh Dickinson University, New Jersey.

“Cellular intergenomic interaction: A tale of two genomes”. 2000 - Graduate program research lecture, Department of Biology, Farleigh Dickinson University, New Jersey

Research Highlights:

Developed cellular models for study of mitochondrial diseases.

1. Cybrid Technology

In association with Dr. Michael King, set up the cybrid technology at the Center in Dr. Eric Schon's laboratory for the study of mtDNA mutations. Established cybrid cell lines harboring a wide range of point mutations and rearrangements from patient samples referred to the Center which provided a valuable research tool for several postdoctoral fellows and graduate students.

2. Mouse ρ^0 cell line

After several years, succeeded in establishing a mouse ρ^0 cell line, LMTK⁻17, using the DNA intercalating drug ditercalinium (Clark et al 2002).

3. *Human ventricular cardiomyocyte cell line*

Generated a proliferating human ventricular cardiomyocyte cell line by a novel mitochondrial function-based approach. The method can be used generically to transform virtually all postmitotic cells and force them to reenter the cell cycle. The cardiomyocyte cell lines express cardiomyocyte phenotype and have been manipulated to differentiate in culture, by the silencing of the SV-40 oncogene by the siRNA technology. Both the technique of immortalization and the cardiomyocyte cell line are covered under a U.S. patent application. This is the only available proliferating human ventricular cardiomyocyte cell line.

4. *Nuclear swap technology*

Developed the nuclear swap technique to generate transnuclear fibroblasts (Giordano et al 2002). By this method it is possible to generate reconstituted cells containing various combinations of virtually any nuclear and cytoplasmic backgrounds. These models will allow testing of the nuclear contribution in patients with homoplasmic mtDNA mutations associated with expression of tissue-specific phenotypes.

5. *A4300G mtDNA mutation*

Established cardiomyocyte cultures harboring the cardiomyopathy-specific A4300G mtDNA mutation from transplanted heart tissue from Dr. Giulia d'Amati's patient in Rome. Currently, we are studying cardiomyocytes with this mutation in normal and patient nuclear background to define the role of any nuclear factor that may act synergistically with the mtDNA mutation to cause the disease.

6. *Astrocytes and Purkinje neuron studies in MELAS*

Previously, we isolated Purkinje neurons from an autopsied brain tissue from a patient with MELAS, and maintained in culture for several weeks. A study of the mutation in single Purkinje cells revealed a constant, but high level of the MELAS mutation (89%) in every neuron that was studied. Astrocyte cultures from the same patient showed a higher (96%) mutation load. The astrocytes will be used in conjunction with endothelial cells for blood-brain barrier studies.

7. *Intermitochondrial complementation*

Demonstrated that mitochondria can interact resulting in functional complementation of non-overlapping deletions in proliferating cybrid cells. In association with Dr. Michael King in Dr. Schon's laboratory, I developed the method of repopulating virtually any postmitotic cell culture with exogenous mitochondria by treating human myoblasts with R6G, a mitotoxin which selectively renders the mitochondria nonviable. The R6G-treated myoblasts are rescued by fusion with cytoplasts containing exogenous mitochondria with or without mutations. Using this model, we showed that rescue of respiratory chain defect due to deletions and point mutations can occur in postmitotic muscle. These data contribute to our understanding of mtDNA interactions and have therapeutic implications.

8. *In vitro model of the blood-brain barrier*

Propose to study the blood-brain barrier permeability in MELAS using an *in vitro* co-culture model comprised of normal endothelial cells on one side of the membrane and normal astrocytes and neuronal cells on the other. Preliminary co-cultures using normal cells were established, which exhibited high transendothelial cell resistance consistent with the establishment of a tight barrier.

Research Collaborations

In addition to collaborations with other PIs at the Center, active collaborations with:

1. Dr. Tom Hei, Professor of Radiation Research, Columbia University.
Studies of genotoxicity of arsenic and asbestos via mitochondrially mediated free radical production.
2. Dr. Hal Skopicki, Assistant Professor of Medicine, Columbia University and Director of Heart Failure Center, Northshore University Hospital, Long Island.
Studies of transcription factors in cardiac development using a human ventricular cardiomyocyte cell line.
3. Dr. Douglas Turnbull and Dr. Robert Taylor, University of New Castle, UK.
Studies of the A4300G mutation.
4. Dr. Giulia D'Amati and Dr. Carla Giordano of La Sapienza, Rome.
Studies of cardiomyopathy associated with the A4300G mutations.

Manuscript Reviews

Ad hoc reviewer for the following journals:

AJHG, Genetics, BBA, J. Neurol. Sci., Internal Medicine J., Mitochondrion, Muscle and Nerve, Cellular and Molecular Biology, Human Molecular Genetics, Laboratory Investigations.

CU-related activities

Columbia University Senator, Elected 2003

Active member, Research Officers' Committee, Columbia University.

Publications:

Original papers

**Senior and Corresponding author*

1. Valmikinathan, K., **Mascreen, M.**, Meenakshisundaram, E., and Snehalatha, C. (1973) Biochemical Aspects of Motor Neuron Disease - Madras Pattern. J.Neurol. Neurosurg. Psychiat. 753-756.
2. **Mascreen, M.**, Biswakumar, B., and Valmikinathan, K. (1975) Serum Factors Influencing creatine phosphokinase. *In Vitro* studies using diffusates. J.Neurol.Sci. 25, 389-396.
3. Sayeed, Z.A., Velmurugendran, C.U., Arjundas G., **Mascreen, M.**, and Valmikinathan, K., (1975) Anterior Horn cell disease seen in south India. J.Neurol. Sci. 26, 489-498.
4. Sayeed, Z.A., **Mascreen, M.**, Arjundas, G., and Valmikinathan, K. (1976) Plasma lactate in Anterior Horn cell disease. J.Neurol.Sci. 29, 1-7.

5. **Davidson, M.**, Miranda, A.F., Bender, A.N., DiMauro, S., and Vora, S. (1983) Muscle Phosphofructokinase deficiency - Biochemical and Immunological studies of phosphofructokinase isozymes in muscle culture. *J.Clin.Invest.* 72, 545-550.
6. **Davidson, M.**, Collins, M., Byrne, J., and Vora, S. (1983) Alterations of Phosphofructokinase isozymes during human development. Establishment of adult organ specific patterns. *Biochem. J.* 214,703-710.
7. Vora, S., **Davidson, M.**, Seaman, C., Noble, N.A., Tanaka, K.R., Frenkel, E.P., DiMauro, S. (1983) Heterogeneity of the molecular lesions in inherited phosphofructokinase deficiency. *J.Clin.Invest.* 72,1955-2006.
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9. **Davidson, M.**, Yoshidome, H., Stenroos, E.S., and Johnson, W.G. (1991) Neuron-like cells in culture from tuberous sclerosis tissue. *Ann.N.Y.Acad.Sci.* 615,196-210.
10. Johnson, W.G., Yoshidome, S., Stenroos, E.S., **Davidson, M***. (1991) Origin of the neuron-like cells in tuberous sclerosis tissues. *Ann. N. Y. Acad. Sci.* 615, 211-219.
11. King, M. P., Koga, Y., **Davidson, M.** and Schon, E. A. (1992). Defects in mitochondrial protein synthesis and respiratory chain activity segregate with the tRNA^{Leu(UUR)} mutation associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes. *Mol. Cell. Biol.* 12, 480-490.
12. Schon, E.A., Koga, Y., **Davidson, M.**, Moraes, C.T. and King, M.P. (1992). The mitochondrial tRNA^{Leu(UUR)} mutation in MELAS: a model for pathogenesis. *Biochim. Biophys. Acta* 1101, 206-209.
13. Koga, Y., **Davidson, M.**, Schon, E.A. and King, M.P. (1993). Fine mapping of mitochondrial RNAs derived from the mtDNA region containing a point mutation associated with MELAS. *Nucleic Acids Res.* 21, 657-662.
14. Masucci, J.P., **Davidson, M.**, Koga, Y., Schon, E.A. and King, M.P. (1995). In vitro analysis of mutations causing myoclonus epilepsy with ragged-red fibers in the mitochondrial tRNA^{Lys} gene: two genotypes produce similar phenotypes. *Mol. Cell. Biol.* 15, 2872-2881.
15. Koga, Y., **Davidson, M.**, Schon, E.A. and King, M.P. (1995). Analysis of cybrids harboring MELAS mutations in the mitochondrial tRNA^{Leu(UUR)} gene. *Muscle and Nerve Suppl.* 3, S119-S123.
16. Sobreira, C., **Davidson, M.**, King, M.P. and Miranda, A.F. (1996). Dihydrorhodamine 123 identifies impaired mitochondrial respiratory chain function in cultured cells harboring mtDNA mutations. *J. Histochem. Cytochem.* 44, 571-579.
17. Hirano, M., Shtilbans, A., Mayeux, R., **Davidson, M.**, DiMauro, S., Knowles, J., Schon, E.A. (1997). Apparent mtDNA heteroplasmy in Alzheimer's Disease patients and in normals due to PCR amplification of nucleus-embedded mtDNA pseudogenes. *Proc Natl Acad Sci USA* 94:14894-14899
18. DiMauro, S., Bonilla, E., **Davidson, M.**, Hirano, M., and Schon, E.A. (1998). Mitochondria in neuromuscular disorders. *Biochim. Biophys. Acta.* 1366, 199-210.

19. Brini, M., Pinton, P., King, M.P., **Davidson, M.**, Schon, E.A., and Rizzuto, R. (1999). A calcium signalling defect in the pathogenesis of a mitochondrial DNA inherited oxidative phosphorylation deficiency. *Nat. Med.* 5, 951-954.
20. Papadapoulou L.C., Sue C.M., **Davidson, M.**, Tanji, K, Nishino, Ichizo, Sadlock, JE, Krishna, S, Walker, W, Selby, J, Glerum, DM, Van Coster, R, Lyon, G, Scalais, e, labal, R, Kaplan, P, Shanske, S, De Vivo, D, Bonilla, E, Hirano, M, DiMauro, S and Schon, EA. (1999) Fatal infantile cardioencephalomyopathy with COX deficiency and mutations in *SCO2*, a COX assembly gene. *Nature Genetics*: 3, 333-337.
21. Carrozzo, R., **Davidson, M.**, Walker, W., Hirano, M and Miranda, A.F. (1999). Cellular and molecular studies in cell cultures from patients with multiple mitochondrial DNA deletions. *J.Neurol. Sci.* 170, 24-31.
22. Claudia Sobreira, Michael P.King, **Mercy M. Davidson**, Hyejong Park, Yasutoshi Koga and Armand, F. Miranda. Long-term analysis of differentiation in human myoblasts repopulated with mitochondria harboring mtDNA mutations. (1999) *Biochem. Biophys. Res. Commun.* 266, 179-186.
23. Hoffbuhr, KC, Davidson, E, Filiano, BA, **Davidson, M.**, Kennaway, NG and King, MP.(1999) A pathogenic 15-base pair deletion in mitochondrial DNA-encoded cytochrome *c* oxidase subunit III results in the absence of functional cytochrome *c* oxidase. *J. Biol. Chem.*275: 13994-14003.
24. Karadimas, CL, Greenstein,,P, Sue, CM, Joseph, JT, Tanji, K,Haller, RG,Taivassalo, T, **Davidson, MM**, Shanske, S, Bonilla, E, and DiMauro, S. (2000). Recurrent Myoglobinuria due to a nonsense mutation in the *COX I* gene of mitochondrial DNA. *Neurol*, 55, 644-649.
25. Hirano M, **Davidson M**, DiMauro S. (2001). Mitochondria and the heart. *Curr Opin Cardiol*; 16: 201-210.
26. Leonardo Salviati, Evelyn Hernandez-Rosa, Winsome F. Walker, Sabrina Sacconi, Salvatore DiMauro. Eric A. Schon, and **Mercy M. Davidson***. (2002). Copper supplementation restores cytochrome *c* oxidase activity in cultured cells from patients with *SCO2* mutations. *Biochem. J.* 363, 321-327.
27. Carla Giordano, Francesco Pallotti, Winsome F.Walker, Nicoletta Checcarelli, Olimpia Musumeci ,Filippo Santorelli, Giulia d'Amati, Eric A. Schon, Salvatore DiMauro, Michio Hirano, and **Mercy M. Davidson***. Pathogenesis of the deafness-associated A1555G mitochondrial DNA mutation. (2002). *Biochim. Biophys. Res. Commun.* 293, 521-529.
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29. Kim M. Clark, Timothy A. Brown, **Mercy M. Davidson**, Lefkothea C. Papadopoulou and David A. Clayton. (2002). Differences in nuclear gene expression between cells containing monomer and dimer mitochondrial genomes. *Gene* 286, 91-104.

30. Sacconi S, Salviati L, Sue CM, Shanske S, **Davidson MM**, Bonilla E, Naini AB, De Vivo DC, DiMauro S. (2003). Mutation Screening in Patients With Isolated Cytochrome c Oxidase Deficiency. *Pediatr. Res.* 53, 224-230.
31. Robert W. Taylor, Carla Giordano, **Mercy M. Davidson**, Giulia d'Amati, Hugh Bain, Christine M. Hayes, Helen Leonard, Martin-J. Barron, Carlo Casali, Filippo M. Santorelli, Michio Hirano, Robert N. Lightowers, Salvatore DiMauro and Douglass M. Turnbull. (2003). A homoplasmic mitochondrial transfer ribonucleic acid mutation as a cause of maternally inherited hypertrophic cardiomyopathy. *J. Amer. Coll. Cardiol.* 41, 1786-1796..

Manuscript submitted

Novel human cell lines derived from adult human ventricular cardiomyocytes. (2004). **Mercy M. Davidson**, Claudia Nesti, Nithila D. Isaac, Winsome F. Walker, Evelyn Hernandez, Lev Protas, and Michio Hirano.

Su-Xian Liu, **Mercy M. Davidson**, Xiuwei Tang, Mohammad Athar and Tom K. Hei. (2004). Mitochondrial damage mediates genotoxicity of arsenic in mammalian cells.

F. Pallotti, A. Baracca, E. Hernandez-Rosa, W. F. Walker, G. Solaini, G. Lenaz, G.V. Melzi d'Eril, S. DiMauro, E.A. Schon, and **M. Davidson***. (2004). Biochemical analysis of respiratory function in cybrid cell lines harboring mtDNA mutations.

Under Preparation

Davidson, M. Koga, Y., Zhang, L.L., King, M.P., and Schon, E.A. Evidence for genetic and functional complementation of nonoverlapping deletions in human mitochondria.

Sobreira C, **Davidson, M.** Park, HJ, and King MP. Complementation of nonoverlapping deletions in human postmitotic muscle

Walker U, Walker WF, Hernandez-Rosa, E, Schon EA and **Davidson, M***. Mitochondria harboring point mutations can complement to restore function in postmitotic muscle.

Sabrina S, Salviati, L, Hernandez-Rosa, E, Schon, EA, DiMauro, S and **Davidson, M***. C5545Y: A novel functionally dominant mitochondrial DNA mutation.

Nesti, C, Walker, WF, Hernandez-Rosa, E and **Davidson, M***. Silencing the SV-40 oncogene by si RNA results in differentiation of proliferating cardiomyocytes in culture.

Walker WF, Hernandez-Rosa E, Bonilla E, and **Davidson M***. Studies of cultured glial cells and single Purkinje neurons from a patient with the MELAS A3243G mutation.

ESTABLISHMENT OF A HUMAN FETAL CARDIAC MYOCYTE CELL LINE

YI-CHONG WANG, NICOLAS NECKELMANN¹, ANN MAYNE, AHVIE HERSKOWITZ,
ALAGARSAMY SRINIVASAN, KENNETH W. SELL, AND AFTAB AHMED-ANSARI

Department of Pathology and Winship Cancer Center, Emory University School of Medicine, 1327 Clifton Road, N.E., Atlanta, Georgia 30322 (Y.-C. W., N. N., A. M., K. W. S., A. A.-A.), Division of Cardiology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 (A. H.); The Wistar Institute, Philadelphia, Pennsylvania 19104 (A. S.)

(Received 15 June 1990; accepted 10 October 1990)

SUMMARY

Human cardiac myocytes undergo degeneration, cytolysis, and necrosis in a number of clinical disease conditions such as myocarditis, dilated cardiomyopathy, and during episodes of cardiac allograft rejection. The precise cellular, biochemical, and molecular mechanisms that lead to such abnormalities in myocytes have been difficult to investigate because at present it is not possible to obtain and maintain viable cell cultures of human adult cardiac myocytes in vitro. However, human fetal cardiac myocytes are relatively easy to maintain and culture in vitro, but their limited availability and growth, variability from one preparation to another, and varying degrees of contamination with endothelial and epithelial cell types have made it difficult to obtain reliable data on the effect of cardiotropic viruses and cardiotoxic drugs on such myocytes. These thoughts prompted us to attempt to derive a cell line of human cardiac origin. Highly enriched human fetal cardiac myocytes were transfected with the plasmids pSV2Neo and pRSVTag and gave rise to a cell line (W1) which has been maintained in culture for 1 yr. Morphologic and phenotypic analyses of W1 cells by flow microfluorometry and immunoperoxidase techniques indicate that the W1 cell line shares many properties of human fetal cardiac myocytes, but appears not to react with specific antibodies known to react with markers unique to human endothelial, epithelial, skeletal muscle, and dendritic cells. These preliminary data suggest that the W1 cells may provide a unique source of an established cell line that shares many properties ascribed to human cardiac myocytes.

Key words: human cardiac myocyte cell line; transfection.

INTRODUCTION

The difficulties with which adult human cardiac myocytes can be isolated and maintained in vitro (17) have proven to be a major obstacle for comparative studies addressing the function of these cells from normal and diseased individuals. Consequently, it has been virtually impossible to analyze many pertinent pathogenic mechanisms at a cellular, subcellular, and molecular level. Although human fetal cardiac myocytes are relatively easy to maintain in tissue culture (10,15,21,31), their infrequent availability, short life, variability between each primary cardiac cell line, and the varying degrees of contamination of these cell lines with endothelial, epithelial, and dendritic cell types have limited the usefulness of these tissue culture systems for the pathophysiologic study of the action of drugs, cardiotropic viruses, and other agents. With the goal of preparing a permanent source of human fetal cardiac myocytes our laboratory has established such a cell line by cotransfecting a highly enriched population of human cardiac myocytes with the plasmids pRSVTag (encoding the SV40 large T antigen) and the pSV2Neo (containing a neomycin resistance gene) (26). The resulting W1 cell line has been cloned by limiting dilution and neomycin selection and has been maintained in culture for the past 18 mo. The present communication describes the cell line's growth

characteristics and phenotypic characterization. Data from these studies demonstrate that the W1 cell line possesses a significant number of properties that are ascribed to cardiac myocytes. The results also distinguish this cell line from skeletal muscle, endothelial, and epithelial cell lines. Thus, it is anticipated that as a surrogate, the W1 cell line will provide a unique tool for studies that will focus on the pathophysiology of human cardiac myocytes.

MATERIALS AND METHODS

Preparation of enriched populations of human fetal cardiac myocytes. The procedures used to prepare enriched populations of human fetal cardiac myocytes have been described in detail elsewhere (31). Briefly, the fetal heart was excised from a freshly procured product of conception. After carefully trimming away the aorta and connective tissue, etc., the remaining heart tissue was minced with sterile scalpel blades. The tissue fragments and cells were resuspended in basal medium Eagle (BME) (GIBCO, Grand Island, NY) containing 20% fetal bovine serum (FBS) (Hyclone Labs, Logan, UT) and centrifuged at 150 $\times g$ for 10 min at room temperature. The cell pellet was then resuspended in 2 ml BME containing 0.3% collagenase (Worthington Biochemicals, Freehold, NJ) by gentle vortexing for 8 min at room temperature. The larger tissue fragments were allowed to settle, and the supernatant fluid containing the cells was transferred to a new centrifuge tube.

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The collagenase treatment of the tissue fragments and the collection of the dissociated cells were repeated 3 times. The resulting cells were washed twice with BME medium containing 20% FBS, resuspended in 6 ml of myocyte culture medium (described below), layered onto a discontinuous isotonic 45, 60, and 70% Percoll gradient (Pharmacia, Piscataway, NJ), and then centrifuged at $450 \times g$ for 30 min. The cells at the 45 to 60% interface were collected, washed twice with the myocyte culture medium, and then incubated in 60-mm sterile polystyrene petri dishes for 1 h at 37°C . Nonadherent cells were removed and incubated again in fresh petri dishes, and this procedure was repeated twice. Finally, the nonadherent cells were incubated for 1 h at 37°C in a petri dish previously coated with antibodies specific for the human common leukocyte antigen CD45 (Becton-Dickinson, Mountain View, CA) for the removal of potentially contaminating leukocytes. The final batches of nonadherent cells (usually about 1.5×10^5 viable cells/ml) were used for transfection experiments as described below.

The purity and the identity of the cells were verified with a monoclonal antibody (clone 1H1, previously generated in our laboratory) which specifically reacts with human cardiac myosin. An aliquot of the cells was treated with a predetermined optimal concentration of fluorescein conjugated anti human MHC class I 7.4 monoclonal antibody (clone W6/32, Serotec Labs, Indianapolis, IN) or fluorescein conjugated anti-human MHC class II monoclonal antibody (clone L-243, American Type Culture Collection, Rockville, MD), washed, and then fixed in 2% physiologic paraformaldehyde in phosphate buffered saline (PBS), pH 7.4. The cells were then incubated with 0.1 ml of the monoclonal antibody 1H1 (isotype IgM) followed by 0.1 ml of a 1:100 dilution of a phycoerythrin-conjugated goat anti-mouse IgM specific antibody (Southern Biotech, Birmingham, AL). The cells were washed with PBS and then subjected to two-color flow microfluorometric analysis, using a FACS-STAR (Becton-Dickinson). The results showed that >95% of the cells were positive for myosin and expressed low levels of MHC class I, but were negative for MHC class II. Appropriate controls such as the developing antibody alone were included to set gates after the proper forward and right-angle light scatter gates were established, using standard protocols. Previous data from our laboratory have shown that normal human cardiac myocytes do not express detectable levels of MHC class II antigens (1,25), whereas endothelial, epithelial, and dendritic cells, etc., do express varying amounts of such antigens. These data cumulatively demonstrate that the cell populations being used were highly enriched for human cardiac myocytes.

Media for culture of myocytes. A variety of media were tested for optimal growth of human fetal heart cells. These tests led to the selection of culture media and supplements which allowed maximal viability and functional activity (contractility indicative for heart beat) of the myocytes in vitro. The medium of choice suggested by these tests contained 38% BME, 38% Ham's F10 nutrient mixture, 1% L-glutamine (200 mM), 10% heat-inactivated horse serum, 5% heat-inactivated FBS, and 5% human AB serum (all from GIBCO), 2% bovine insulin (50 $\mu\text{g}/\text{ml}$, Sigma Chemical Co., St. Louis, MO), and 1% penicillin-streptomycin (10 000 U/ml penicillin, 1 mg/ml streptomycin, Whittaker MA-Bioproducts, Inc., Walkersville, MD).

Transfection of fetal cardiac myocytes. Fetal cardiac myocytes were cotransfected with the plasmids pSV2Neo and pRSVTag, using the calcium phosphate procedure (16). The medium in the petri

dishes containing cardiac myocytes was replaced with fresh culture medium 30 min before transfection. Plasmid DNA (6 μg of pSV2Neo and 15 μg of pRSVTag per dish) were precipitated with calcium phosphate for 1 h at room temperature by combining 30 μl pSV2Neo, 50 μl pRSVTag, 395 μl double deionized water, and 55 μl 2 M CaCl_2 , and adding this mixture drop by drop to 0.5 ml $2\times$ HeBS ($2\times$ HeBS is made up by combining 8 g NaCl, 0.37 g KCl, 0.125 g Na_2HPO_4 , 1 g glucose, and 5 g HEPES, adjusting the pH to 7.05 to 7.1, and adding double deionized water to 500 ml). In some cases, 2 M CaCl_2 and the $2\times$ DNA precipitation buffer were purchased (5 prime \rightarrow 3 prime Inc., West Chester, PA). The CaPO_4 -DNA-precipitate was added dropwise and evenly to the cells in the culture dishes, and these were then incubated for 4 h at 37°C , 7% CO_2 . After transfection, the cells were shocked with glycerol by removing the medium with the plasmid DNA and replacing it with 4 ml of 15% glycerol in HeBS. After 120 s the cell cultures were carefully rinsed twice with $1\times$ HeBS, and after adding fresh medium, returned to the incubator. Two days later, the transfected cells were selected for neomycin i.e. G-418 resistance by adding 1 mg/ml Geneticin (GIBCO) and growing them in this medium for 2 wk. At this Geneticin concentration, only the transfected cells survived, and it was shown that nontransfected control cells failed to grow in the medium. After the cells reached confluency they were subjected to trypsinization and cloning, using a limiting dilution assay. A cloned cell line was derived and named W1.

The doubling time of the W1 cell line was determined by a) a thymidine uptake assay and b) by cell counts. For the thymidine uptake assay, 10^2 , 10^3 , 10^4 , or 10^5 viable cells (identified by trypan blue dye exclusion assay) were dispensed in a volume of 0.1 ml in triplicate wells of a series of seven 96-well microtiter plates. Individual wells of each plate received 0.02 ml of media containing 1 μCi of methyl- ^3H thymidine (^3H TdR, specific activity 2 Ci/mmol, New England Nuclear, Boston, MA) on Days 0, 1, 2, 3, 4, 5, and 6. Fifteen hours after the addition of thymidine, the cells were harvested with a PHD cell harvester (Cambridge Technology, Inc., Watertown, MA) and the amount of ^3H in each sample was counted with a liquid scintillation counter. The mean uptake of ^3H TdR (and standard deviation) of triplicate samples was calculated. For the cell count assay, total cell counts for each of the triplicates were determined for a similar set of plates.

Cell lines. Several cell lines were used for control purposes. The human lymphoblastoid cell lines CEM (ATCC-CCL119), RAJI (ATCC-CCL86), DAUDI (ATCC-CCL213), and human epithelioid HeLa (ATCC-CCL2) were obtained from the American Type Culture Collection (ATCC) and maintained in the tissue culture media recommended by the ATCC. The human rhabdomyosarcoma cell line A204 (ATCC-HTB2) was also obtained from the ATCC and maintained in the recommended medium. In addition, human umbilical vein endothelial cells (HUVE) were prepared by collagenase treatment as described elsewhere (14,30). The cultures were maintained in RPMI 1640 supplemented with 20% adult bovine serum (Hyclone), penicillin 100 $\mu\text{g}/\text{ml}$, streptomycin 100 $\mu\text{g}/\text{ml}$, and amphotericin B 0.25 $\mu\text{g}/\text{ml}$ (Sigma), heparin 90 $\mu\text{g}/\text{ml}$ (Sigma), and endothelial cell growth supplement, 50 $\mu\text{g}/\text{ml}$ (Collaborative Research, Lexington, MA). HUVE cultures showed typical polygonal endothelial cell morphology and were positively stained by the fluorescent anti-Factor VIII antibodies and the lectin from *Ulex europaeus* [as indicated by staining with the anti-UEAI antibodies (22)]. The HUVE cell lines were serially passaged by brief exposure

to Versene (GIBCO), followed by 0.05% trypsin with 1 mM EDTA in Hanks' balanced salt solution (GIBCO); these primary cell lines usually lasted about 3 to 4 passages. The human lymphoblastoid cell lines were utilized specifically as controls for MHC class I and class II antigen-expressing cell lines. Thus, the RAJI cell line expressed both MHC class I and class II antigens, the CEM cell line only expressed MHC class I but not class II antigens; and the DAUDI cell line expressed MHC class II but not MHC class I antigens.

Quantitation of myosin expression. The relative amount of myosin in human fetal cardiac myocytes, W1 cells, A204 cells, fibroblasts, and endothelial cells was quantitated using myosin-specific rabbit antibodies and radioimmunoassays developed in our laboratory (21). Triplicate cultures of 10^6 viable cells of each cell line were fixed with 4% paraformaldehyde and then assayed for quantitative levels of myosin using $F(ab)_2$ fragments of pan myosin-specific rabbit antibodies (BioGenex Laboratories, Dublin, CA), followed by ^{125}I -labeled $F(ab)_2$ goat anti-rabbit IgG reagent. Controls were set up in a similar manner but included normal rabbit serum in lieu of the myosin-specific antibodies. The same myosin-specific antibodies and control serum were used to stain W1 cells by employing immunoperoxidase techniques (see below).

Light and electron microscopy. Human fetal cardiac myocytes and the W1 cell line were cultured in sterile 8-chamber Tissue-Tek slides (Wilkes Scientific, Naperville, IL) and observed with an inverted Zeiss photomicroscope. Cells were photographed using Kodak technical pan film 2415 (Estar-AH Base). To facilitate photography, the chambers were removed and replaced by cover slips.

For electron microscopy, W1 cells, human fetal cardiac myocytes, and human lung fibroblasts (MRC5, Bartel's Diagnostic Division, Bellevue, WA) were fixed in their tissue culture dishes and processed as described previously (5,19). For control purposes, fetal cardiac tissue was fixed and embedded in the same way. Transverse and en face sections were cut with a Sorvall MT2B ultramicrotome and examined with a JEOL JEM 100C transmission electron microscope.

Enzyme analysis: creatine phosphokinase and lactate dehydrogenase. A total of 10^7 W1 cells and, for control purposes, human fetal cardiac myocytes (human cardiac muscle control) and the A204 human rhabdomyosarcoma cell line (human skeletal muscle control) were suspended in 1 ml of phosphate buffered saline (PBS), pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiopyridine, 1 mM benzamidine, 2.5 μ g/ml each of chymostatin, peptostatin A, leupeptin, elastinal, and antipain, and 5 μ g/ml of aprotinin (Sigma). The cells were then subjected to three freeze-thaw cycles and centrifuged at 750 $\times g$; the supernatant fluids were then sent to the Clinical Pathology Laboratory of Emory University Hospital for analysis of creatine phosphokinase (CPK) and for the relative quantitative expression of the lactate dehydrogenase (LDH) isoenzymes, using standard clinical chemistry procedures. Briefly, CPK isozymes were separated by electrophoresis in cellulose acetate at pH 8.6 and identified by coupling the CPK reaction with the hexokinase and glucose-6-phosphate dehydrogenase (G6PD) reactions to form NADPH, which was visualized by UV light. Patterns of LDH isozyme bands on cellulose acetate strips were visualized using the lactate and pyruvate dehydrogenase reaction in which the NADH formed in the bands reduces the tetrazolium salt to a deeply colored formazan dye which is quantitated by densitometry.

Phenotypic analysis with polyclonal and monoclonal

antibodies. A variety of monoclonal antibodies and reagents previously characterized and known to react with cells of distinct lineages were utilized in attempts to ascertain the lineage derivation of the W1 cell line. Each reagent was used according to the procedures outlined by the commercial vendor or by colleagues who generously donated the reagent (see Table 2 for references). Flow microfluorometric (FMF) techniques were used for the assessment of membrane cell surface proteins on single cell suspensions of each cell line using a FACS-Star. Immunoperoxidase techniques were used to characterize both intracellular and membrane surface proteins of each cell line using the appropriate horseradish peroxidase (HRP) conjugated developing antisera (Vector Labs, Burlingame, CA). FMF techniques were used for testing the reactivity of the monoclonal antibodies 5.1H11, PHM, hec, 14E5, UEAI antibodies, and *Ulex europaeus* lectin (22). Immunoperoxidase techniques were used for detecting the reactivity of polyclonal and monoclonal anti-myosin antibody reagents and reactivities of PAB601 and the UEAI antibodies, PHM, anti-Factor VIII, 14E5, and *Ulex europaeus* lectin after fixing each of the cell lines with 2% paraformaldehyde. For tests with the HHF35 and CGA7 monoclonal antibody reagents (9,29), each cell line was fixed with the cold absolute methanol. Each cell line was tested with three different concentrations of each reagent in three different experiments. The results obtained (see Table 2) are denoted as plus or minus based on the consensus results of all three experiments.

Expression of MHC antigens. Normal human fetal cardiac myocytes were enriched for cardiac myosin-positive cells and suspended in myocyte culture medium at 1×10^6 cells/ml. The W1 cell line was trypsinized, washed 3 times with culture medium, and then suspended at 1×10^6 cells/ml. Aliquots of 1×10^6 cells of each cell type were then dispensed into individual tubes and centrifuged at 150 $\times g$ for 10 min; the supernatant media were discarded. The cells were then resuspended in 0.1 ml of culture medium and incubated with a predetermined optimal concentration of fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-MHC class I antibody (clone W6/32, Serotec Labs) or FITC-conjugated monoclonal anti MHC class II antibody (clone L-243). The control consisted of cells that were incubated with FITC-conjugated normal mouse IgG₁. The monoclonal antibodies utilized were treated with pepsin and then purified by column chromatography to obtain $F(ab)_2$ fragments and then conjugated with FITC before use to prevent nonspecific bonding by Fc fragments. The tubes were incubated for 1 h at 4° C, washed 3 times with PBS (pH 7.4), and then resuspended in 1 ml of PBS (pH 7.4) containing 0.01% sodium azide and 10% FBS. The cells were then subjected to FMF and the frequency and density distribution of MHC class I/II expressing cells determined using a FACS-STAR, after appropriate gating. A total of 10 000 cells per sample were analyzed to obtain the frequency and profile of the relative density of MHC molecules expressed by these cells. Similar techniques were used for cells incubated in media with gamma-interferon (G-INF).

Northern blot analysis for MHC expression. The W1 cell line and human fetal heart tissue were analyzed for the expression of MHC genes by Northern blot analysis. For control purposes we utilized the human CEM, DAUDI, and RAJI cell lines obtained from the ATCC. Total RNA was extracted with RNazol (Cinna/Biotech Laboratories International Inc., Friendswood, TX) based on the guanidine isothiocyanate/acid phenol procedure (3). The RNA was precipitated with isopropanol at -70° C, centrifuged, briefly

dried, and resuspended in 100 μ l 1 mM EDTA. Aliquots of the RNA were denatured, mixed with ethidium bromide, and fractionated in 1.2% agarose 1.8% formaldehyde gels (10 cm long) in a MOPS buffer system (0.04 M MOPS, 0.01 M sodium acetate, 0.001 M EDTA, pH 7.0). Electrophoresis was carried out at 115 V for 90 min. After the gel was photographed, the RNA in the gel was blotted overnight with 20 \times standard saline citrate (SSC) (1 \times SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) onto Hybond N membranes (Amersham, Arlington Heights, IL) by the capillary method. The blots were washed twice for 5 min each in 2 \times SSC and dried for 10 min in an 80° C oven. RNA on the blots was fixed by UV cross linking using a Stratalinker (Stratagene, La Jolla, CA) and the autolink cycle. The RNA blots were then prehybridized overnight at 42° C in Kapak bags (Fisher Scientific) containing the hybridization medium [1 M NaCl, 0.02 M tris, pH 7.4, 3 \times Denhardt's, 100 μ g/ml sonicated salmon sperm DNA, 0.5% sodium dodecyl sulfate (SDS), 50% formamide] (24). Insert DNA from the plasmid HLA-B 7.14 was used to make the MHC class I HLA-B-specific probe, and insert DNA from plasmid T33 was used for the MHC class II HLA-DRA-specific probe. (HLA-B 7.14 and T33 were provided by Dr. Jeremy Boss, Department of Microbiology and Immunology, Emory University). Both probes were generated using alpha-³²P-dCTP (Amersham), and random primer labeling kits (6,7) (Boehringer Mannheim, Indianapolis, IN; United States Biochemicals, Cleveland, OH). For hybridization, 10 \times 10⁶ cpm of probe DNA was denatured and added to the hybridization medium surrounding the RNA blot in the bag, and the incubation at 42° C was then continued overnight. Blots were washed for 30 min in each one of the following solutions at the given temperatures: 2 \times SSC, 0.1% SDS at room temperature; 1 \times SSC, 0.1% SDS at 50° C; 1 \times SSC, 0.1% SDS at 55° C; and 0.1 \times SSC, 0.1% SDS at 60° C. Autoradiographs were obtained using Kodak XAR 5 film (Kodak, Rochester, NY) and intensifying screens at -70° C when necessary.

For control purposes, the RNA blots were rehybridized with a ³²P-labeled oligonucleotide probe specific for human 28S rRNA (2). In these cases, the previous probes were removed from the blots by pouring a boiling solution of 0.1% SDS onto the blots and letting the blots cool to room temperature in this solution. After obtaining autoradiographs to verify whether the probes had been removed, the blots were rehybridized with a 28S rRNA specific probe labeled with ³²P using gamma-³²P-ATP and T4 polynucleotide kinase.

RESULTS

Morphology. The W1 cell line grows in vitro attached to the plastic surface of the culture vessel. Light microscopy showed that W1 cells and human fetal cardiac myocytes (maintained in vitro for 12 d) look very similar (Fig. 1), and these similarities are also confirmed by electron microscopy (Fig. 2). However, there is a decrease in the degree of differentiation of the sarcomeres in the W1 cells. Cardiac tissue is typically more differentiated than fetal myocytes maintained in culture, and these, in turn, seem to be more differentiated than the W1 cells (in these comparisons, the degree of organization of the sarcomeres is used as an indicator of the degree of differentiation). Figure 2 illustrates some of the ultrastructural features that are found in W1 cells and cardiac myocytes but not in fibroblasts (data not shown).



Fig. 1. Photomicrographs of W1 cells (a) and human fetal cardiac myocytes maintained in vitro for 12 d (b). Note that by morphologic criteria these two types of cells are indistinguishable from one another. $\times 210$.

Growth characteristics. The growth curve of W1 cells was determined by using [³H]TdR uptake assays and by cell counts. The results obtained with these two methods are similar and are presented in Fig. 3. Statistical analysis of the data for 10⁴ input cells shows that the doubling time for the W1 cell line is about 55.4 h. When 10³ input cells were used, there was a lag, and 10⁵ cells were too many for optimal growth in the 96-well microtiter plates.

Initially, Geneticin was used to select for G-418 resistance, but subsequently the W1 cells were grown in media without Geneticin. To determine whether the neomycin resistance persists, Geneticin was again added to the culture medium 5 mo. after the transfection, and it was shown that the cells were still resistant to the drug.

Phenotypic characterization of the W1 cell line. Results of the CPK and LDH analyses showed that the W1 cells contain the same isoenzyme pattern as fetal cardiac myocytes and distinct from that seen with the A204 human rhabdomyosarcoma cell line. Radioimmunoassays were employed to assess and compare the myosin content of human fetal cardiac myocytes, the W1 cell line, the human rhabdomyosarcoma cell line A204, fibroblasts, and endothelial cells. The results of these assays are presented in Table 1 and clearly indicate that human fetal cardiac myocytes, W1 cells and A204 cells contain much more myosin than the fibroblasts or endothelial cells. The data obtained with the control antibodies also show



FIG. 2. Electron micrographs of W1 cells (a) and human fetal cardiac myocytes (maintained in vitro for 3 d) (b). Myofibrils (M) and disorganized Z-band (Z) can be identified in both W1 cells and native cardiac myocytes. X59 000.

that more of the myosin-specific antibodies reacted with all five cell types.

A series of monoclonal and polyclonal antibodies was used to characterize the cardiac myocyte lineage derivation of the W1 cell line, and in efforts to distinguish the cell line from endothelial and other cell types. Specificity of the reagents was verified by testing fresh, enriched human fetal cardiac myocytes, human endothelial cells, a human epithelial cell line, human dendritic cells enriched as previously described (33), and the A204 cell line. Each of these cell lines was examined in parallel with the W1 cell line. The reactivities of the sera were examined on either a single cell suspension of each cell line, using flow microfluorometry, or after fixation of the cells cultured in Tissue-Tek slides following standard immunoperoxidase techniques. Each reagent was titrated on the cell line that was previously shown to react with this reagent, and three dilutions (the optimal and twofold lower or higher dilution) were used to stain each of the cell lines assayed by each technique. As seen in Fig. 4 and Table 2, the W1 cell line and fresh human cardiac myocytes react specifically with murine monoclonal antibodies 1H1 and 17B11, which are directed against human cardiac myosin. These two monoclonal antibodies do not react with human skeletal muscle myosin and were shown not to react with the A204 cell line and the human

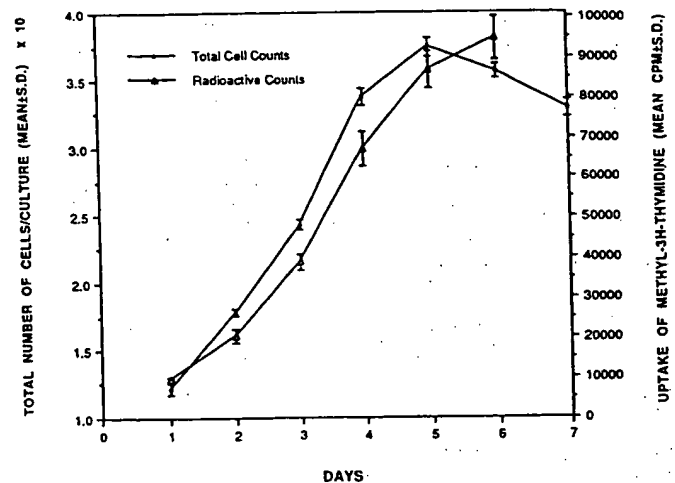


FIG. 3. In vitro growth characteristics of the W1 cell line (doubling time). The W1 cell line was cultured in 96-well microtiter plates at 10^2 , 10^3 , 10^4 , and 10^5 cells in a volume of 0.1 ml of myocyte culture medium. Cultures at each cell concentration were performed in triplicate. Replicate plates were either pulsed with [3 H]thymidine and cultures harvested on Days 1 to 7, or total viable counts were performed of cells in each individual well. Data above represent the mean cell counts and mean uptake of [3 H]thymidine of triplicate cultures containing 104 initial input cells.

epithelial cell line and human endothelial and dendritic cells. The W1 cell line also reacted with other anti-myosin monoclonal antibody reagents 5D1, 9A4, CC52, and a polyclonal anti-myosin reagent.

A typical result obtained with a myosin-specific rabbit antiserum is shown in Fig. 5. The W1 cell line reacted with HHF35 but not with CGA7 anti-actin monoclonal reagents; it also failed to react with 5.1H11 monoclonal antibody reagent, which detects a determinant on human skeletal muscle myoblasts (32). W1 cells also failed to react with a monoclonal reagent PHM, which reacted with endothelial cells but not the A204 cell line. In addition, the W1 cell line failed to react with a series of reagents that have been previously characterized to react with endothelial cells (hec, 14E5, polyclonal anti Factor VIII, and the *Ulex europaeus* lectin), suggesting that the lineage of the W1 cell line is distinct from endothelial cells. Finally, the W1 cell line did react with a monoclonal anti-SV40 Tag reagent (8), indicating that the DNA encoding the SV40 large antigen, which was utilized to transfect human fetal cardiac myocytes, was present in the resultant W1 cell line. These data cumulatively sug-

TABLE 1

QUANTITATION OF MYOSIN CONTENT IN THE W1 CELL LINE

Cell Line	RIA Results 125 I-cpm (Mean \pm S.D.)	
	Control, Normal Rabbit Serum	Experimental, Anti-myosin Antibodies
Human fetal cardiac myocytes	236 \pm 47	32647 \pm 1573
W1	414 \pm 62	25683 \pm 3137
A204	1374 \pm 77	28924 \pm 4019
Fibroblast	723 \pm 68	4274 \pm 389
Endothelial	662 \pm 59	3266 \pm 411

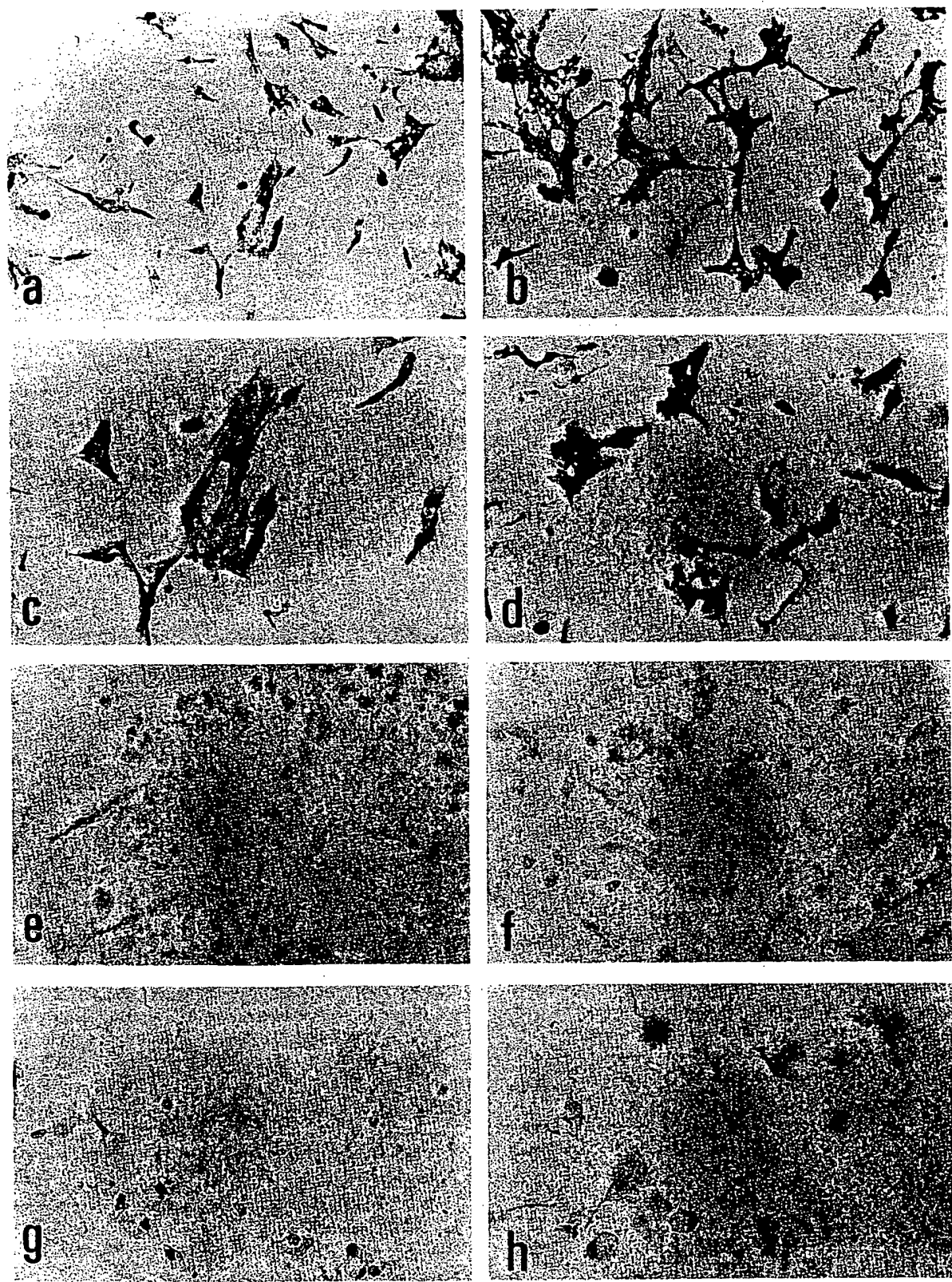


FIG. 4. Photomicrographs of cells stained with the cardiac myosin-specific monoclonal antibodies 1H1 (a, c, d, and g) and 17B11 (b, d, f, and h) visualized by immunoperoxidase techniques. W1 cells (a and b) and human fetal cardiac myocytes (c and d) react with these antibodies, but A204 cells (e and f) and human umbilical vein endothelial cells (g and h) do not.

TABLE 2
REACTIVITIES WITH MONOCLONAL AND POLYCLONAL ANTIBODIES

Reagent or Monoclonal Antibody	Antigen Spec. (Ref.)	Reactivity With,				
		W1 Cells	Fetal Cardiac Myocytes ^a	A204 Cells	HUVE Endothelial Cells ^a	HeLa Epithelioid Cell Line
1H1 ^b	myosin	+	+	-	-	-
17B11 ^b	myosin	+	+	-	-	-
5D1 ^b	myosin	+	+	+	+	±
9A5 ^b	myosin	+	+	+	+	±
CC52	cardiac myosin (Dr. Radovan Zak)	+	+	+	ND	+
Anti-myosin (polyclonal)	skeletal muscle myosin (Miles Scientific)	+	+	+	ND	+
Anti-myosin (polyclonal)	myosin (BioGenex Laboratories)	+	+	+	ND	+
HHF35	cardiac, skeletal, and smooth muscle actin (29)	+	+	+	-	+
CCA7	smooth muscle actin (9)	-	-	-	-	-
5.1H11	human skeletal muscle myoblasts (32)	-	-	+	-	-
PHM	human epithelium and endothelium (11) (Australian Monoclonal Development)	-	-	-	+	+
hec	human endothelial cell (20)	-	-	-	+	-
Anti-factor VIII (polyclonal)	endothelial cell marker (Signet Laboratories, Dedham, MA)	-	-	-	+	-
14E5	human endothelial (ATCC-HB174)	-	-	-	+	-
Ulex europaeus lectin and UEA1 Abs	endothelial cell marker (22) (Vector Laboratories, Burlingame, CA)	-	-	-	+	-
PAb601	SV40 TA _g (8)	+	-	-	-	-

^a Fresh, enriched human primary fetal cardiac myocyte cells and primary HUVE cells obtained using methods described in Materials and Methods (14).

^b Hybridomas generated in our laboratory.

gest that the W1 cell line has surface antigens that are similar to those expressed by cardiac myocytes but unique and different from those expressed by the A204 cell line, the epithelial cell line, and the primary human endothelial cell lines, consistent with a cardiac myocyte origin of the W1 cell line. The data obtained using immunoperoxidase and flow microfluorometric techniques essentially gave similar profiles for each reagent and cell line.

Expression of MHC antigens. One of our goals is to use the W1 cell line as a surrogate for human cardiac myocytes in efforts to gain insights on the humoral and cellular effector mechanisms in patients with myocarditis or dilated cardiomyopathy and in patients undergoing transplant rejection. The cellular effector mechanisms have been shown to be mediated by recognition of MHC class I and class II antigens by host effector cells on target tissues. These thoughts prompted us to examine the expression of such MHC class I and II antigens in the W1 cell line. Studies from our laboratory (1) and others (23,27,28) have previously shown that normal human adult cardiac myocytes do not express detectable levels of MHC class II antigens nor very low levels of MHC class I antigens. This was also true for normal human fetal cardiac myocytes. These myocytes, however, express high levels of both MHC class I and class II antigens in cardiac biopsy tissues of patients undergoing rejection episodes posttransplantation (27,28) and in cardiac tissues of patients with myocarditis (12). Furthermore, human fetal cardiac myocytes cultured in vitro with cytokines, notably G-INF, were shown also to lead to marked increases in the levels of MHC class I and class II expression.

The W1 cell line shows a diffuse pattern of staining with anti-hu-

man cardiac myosin, and 100% of these cells stain with these reagents (data not shown). In contrast, preparations of normal human fetal cardiac myocytes show varying frequencies of myosin-positive cells (45 to 70%). A variety of procedures were used to enrich for cardiac myosin-positive human fetal cardiac myocytes. This included density gradient purification and the property of differential adherence of myocytes as compared with the other contaminating cells. After such enrichment procedures, human cardiac myocytes can be enriched such that >90 to 95% of the cells stain with anti-human cardiac myosin. These procedures were utilized, and such enriched normal human fetal cardiac myocytes were examined for expression of MHC class I and II antigens in parallel with the W1 cell line.

As seen in Fig. 6, the enriched normal human fetal cardiac myocytes fail to express MHC class II antigens (Fig. 6 C) but do express low levels of MHC class I antigens (Fig. 6 B). It was of interest that the W1 cell line similarly showed low levels of MHC class I antigen expression and failed to show MHC class II antigen expression. In efforts to determine if the expression of such MHC class I and II antigens can be induced in vitro, cultures of the W1 cell line and enriched normal human cardiac myocytes were incubated in vitro with varying concentrations (10^5 to 10^8 U of human G-INF for varying days (Days 1 to 7). These cultures were then examined daily for plasma membrane expression of MHC class I and II antigens. As little as 12 500 U/ml of G-INF induced marked increases in the expression of both MHC class I and class II antigens by Day 4 in both the W1 cell line and the normal human fetal cardiac myocytes (Fig. 6). Increasing concentrations of G-INF or longer incuba-

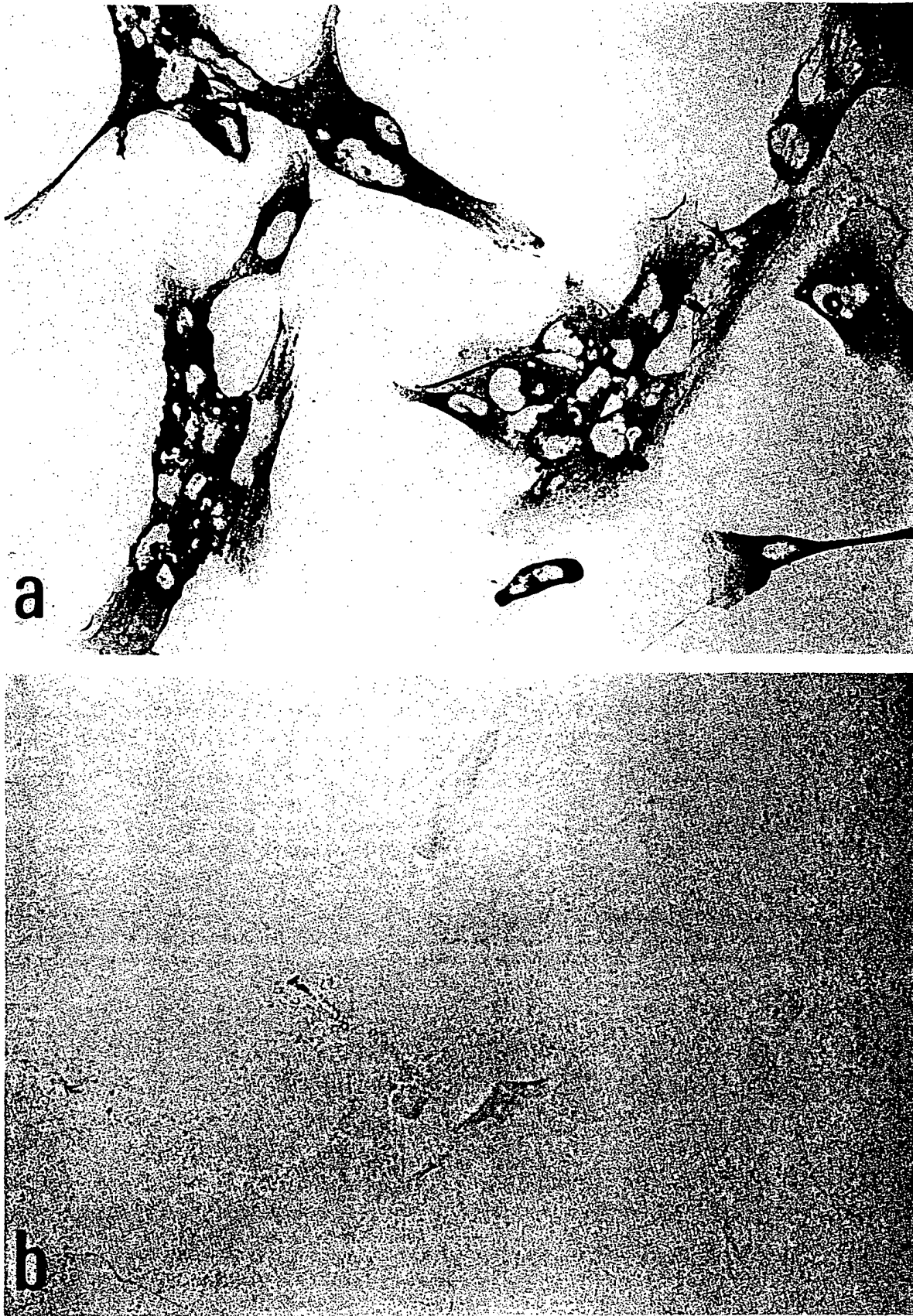


FIG. 5. Photomicrographs of W1 cells stained by using immunoperoxidase techniques with myosin-specific rabbit antibodies (a) or normal rabbit serum (b). $\times 200$.

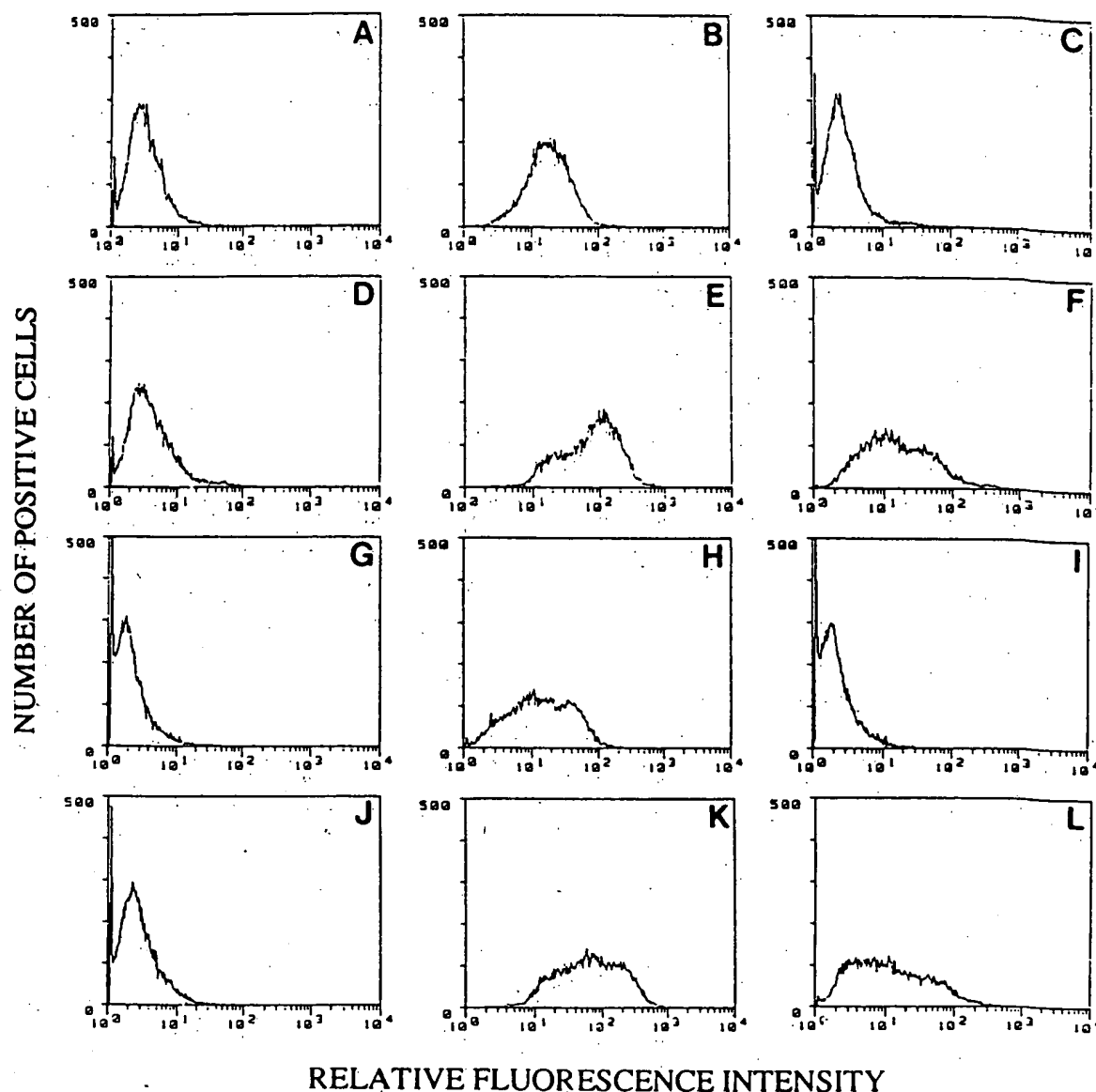


Fig. 6. Comparative FMF profile of cell membrane expression of MHC class I and II antigens on normal human fetal cardiac myocytes before (A-C) and after incubation with G-INF (D,E,F) and the W1 cell line before (G,H,I) and after incubation with G-INF (J,K,L). Aliquots of each cell type were stained with either FITC anti-mouse Ig (control) (A,D,G,J) or predetermined optimum concentrations of FITC F(ab)₂ fragments of monoclonal anti-MHC class I (B,E,H,K) or fluorescein conjugated F(ab)₂ fragments of monoclonal anti MHC class II (C,F,I,L) antibodies.

tions essentially gave similar results (data not shown). These data strongly suggest that the W1 cell line shares this characteristic of MHC class I and II expression with normal human fetal cardiac myocytes.

Tissue typing. With thoughts of using this cell line to study cell-mediated immune responses against virus-infected or drug-treated cells, and with the knowledge that such events are MHC restricted, the W1 cell line was subjected to tissue typing for MHC class I and class II antigens. Native W1 cells could only be phenotyped for MHC class I antigens since no MHC class II antigens could be detected (Fig. 6). Tissue typing showed that the W1 cell line expresses HLA-A2, A30, B17, 18, BW4.6 and CW2.3. To determine the identity of MHC class II antigens, the W1 cells were

first incubated in vitro with G-INF and then subjected to phenotype analysis for both MHC class I and MHC class II antigens. The MHC class I phenotype was the same as for the native W1 cells, with an apparent increase in the sensitivity for the typing of MHC class I antigens. MHC class II typing was only able to detect HLA-DR3 and DRw52.

Northern blot analyses. Although data above (Fig. 6) clearly demonstrated that human fetal cardiac myocytes and the W1 cell line can be induced to express increased levels of MHC class I and II antigens when cultured in vitro with G-INF, it was reasoned that such expression could be secondary to changes induced on the membranes of these cells. Such changes could result in alteration of these proteins in a form that is more readily detected by monoclonal

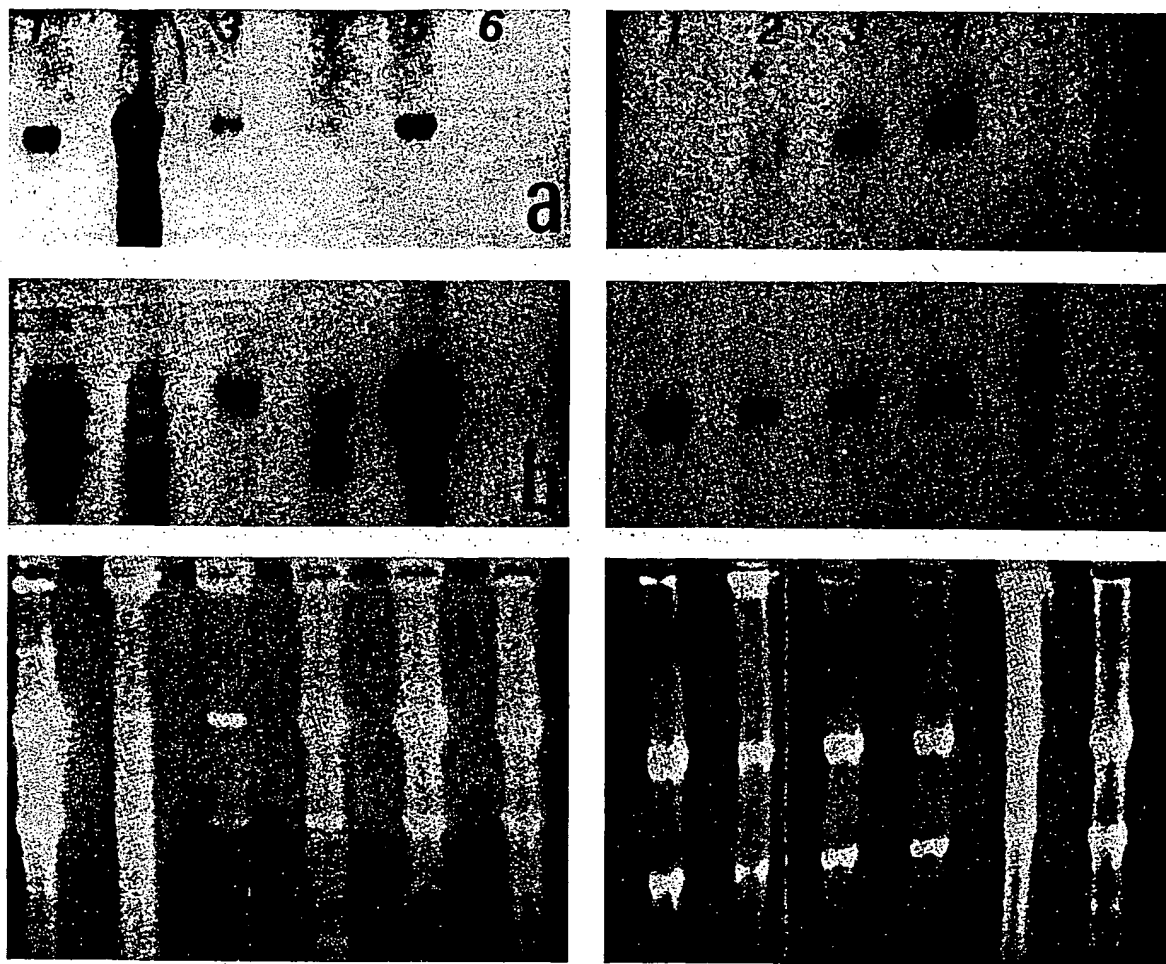


FIG. 7. Northern blot analysis of total RNA from different cell lines. W1 cells (lane 1), W1 cells incubated in a medium containing G-INF (lane 2), DAUDI cells (lane 3), RAJI cells (lane 4), CEM cells (lane 5), and mouse cells (lane 6). Autoradiographs of a Northern blot hybridized with the MHC class I and class II specific probes are shown in a and d, respectively; b and e show that these blots had hybridizable RNA (in lanes 1-5) and that the 28S rRNA specific oligonucleotide probe (lane 2) did not hybridize to mouse RNA (lane 6). Integrity and amounts of total RNA loaded in each lane of the agarose gels which were used for the two blots are shown in c and f; in the presence of ethidium bromide, the 28S and 18S rRNAs are clearly distinguishable.

anti-MHC class I and II antibodies and not due to increased synthesis. To address this issue, RNA was extracted from W1 cells and from W1 cells which were cultured *in vitro* with G-INF for 4 d. To identify such transcripts, blots of total RNA from the cells were prepared and hybridized with the MHC class I HLA-B-specific probe HLA-B 7.14 insert probe or with the MHC class II DRA-specific probe T33 insert probe (see Materials and Methods). The results of these hybridization experiments are presented in Fig. 7. Ethidium bromide staining showed that similar amounts of RNA were loaded in each lane (Fig. 7 C and F), and hybridization of the oligonucleotide probe specific for 28S ribosomal RNA indicates that on each filter comparable amounts of hybridizable RNA were present in each lane (Fig. 7 B and E). The probes react specifically since they did not hybridize with mouse RNA. The MHC class I probe hybridized with RNA from the native W1 cell line, in addition to RNA from the DAUDI, RAJI (to a minor extent), and CEM cells, as expected. The MHC class II probe hybridized with RNA only from W1 cells cultured *in vitro* with G-INF for 4 d but not with RNA

from the native W1 cell line. In addition, as expected, the MHC class II probe hybridized with RNA from the DAUDI and RAJI cells. A comparison of signal intensities obtained with the MHC class I and class II probes (Fig. 7 A and D) clearly indicates that W1 cells express increased amounts of message for MHC class I and newly synthesized MHC class II transcripts when cultured with G-INF. Thus, in W1 cells, MHC class II transcripts were only detected after incubation of the cells for 4 d in media containing G-INF, consistent with data obtained using flow microfluorometric techniques (Fig. 6).

DISCUSSION

A thorough experimental analysis of the cellular and molecular mechanisms involved in the pathophysiology of human cardiac diseases is severely limited by the difficulties of procuring and maintaining human cardiac myocytes *in vitro*. To date, no other human cardiac myocyte cell line is available. Here we have reported how,

for the first time, transfection techniques were used to generate human fetal cardiac myocyte cell lines. The W1 cell line is one of the resultant cell lines and has been shown to share morphologic and phenotypic characteristics with native human fetal cardiac myocytes maintained in vitro. Southern blot analysis of genomic DNA has indicated that both pSV2neo and pRSVTag DNA are integrated into the W1 cells' genome (data not shown), and these findings are corroborated by the fact that the cells are resistant to Geneticin (conferred by the neomycin, i.e. G418 resistance marker on the pSV2neo plasmid) and express the SV40 large T antigen encoded by pRSVTag.

Morphology. Human fetal cardiac myocytes which have been maintained in vitro for 12 d and W1 cells have similar morphologies (Figs. 1 and 2). Both cell types are no longer differentiated and in this way differ significantly from native human cardiac myocytes. This lack of differentiation is also discernible by electron microscopy. For example, myofibrils and Z bands are clearly distinguishable in cells that have just been isolated from fetal heart tissue, but after several days in vitro these organized myofibrils disappear. At this stage, only disorganized bundles (presumably consisting of myosin) are found in fetal cardiac myocytes and, to a lesser extent, in W1 cells (Fig. 2). Because of this lack of differentiation, it is clear that the W1 cell line will have to be utilized with these limitations in mind.

The expression of CPK and LDH isoenzyme patterns were analyzed after 3, 6, and 12 mo. of in vitro culture of the W1 cell line. At each time interval, the pattern seen with the W1 cell line was shown to be characteristic for cardiac cells. Thus, these results are consistent with the concept that the W1 cell line is stable and most likely represents a cell line of a cardiac myocyte lineage.

Expression of intracellular proteins. The similarities between W1 cells and cardiac myocytes were also substantiated by testing reactivities of a variety of antibodies which specifically react with proteins in cardiac myocytes (Tables 1 and 2, Fig. 4). The reactivities with myosin are especially notable. Thus, both the radioimmunoassay data (Table 1) and the immunoperoxidase techniques (Fig. 4 and 5) support our conclusion that the W1 cell line really is a cardiac cell line. The specific reactivities of our monoclonal antibodies 1H1 and 17B11, which specifically react with cardiac myosin (Table 2 and Fig. 4) with W1 cells, also corroborate this conclusion. Antibodies and the *Ulex europaeus* lectin, which are known to react with endothelial cells or fibroblasts, failed to react with W1 cells, indicating that the W1 cells lack these specific cell markers. Similar experiments employing appropriate controls and antibodies raised against actin, fibronectin, and the fibroblast cell markers vimentin and cytokeratin showed that W1 cells do not react with these reagents. Furthermore, it was found that, unlike endothelial cells, the W1 cells did not respond to the addition of endothelial cell growth factor (ECGF) (Chemicon International Inc., El Segundo, CA). In summary, all these experiments indicate that the W1 cells are neither fibroblasts nor endothelial cells. In other preliminary experiments (data not shown) it was found that W1 cells are highly susceptible to infections by cardiotropic viruses such as the CVB3 coxsackievirus; thus, in this respect these cells also resemble cardiac myocytes.

MHC class I and class II expression is similar to cardiac myocytes. The mean density of MHC class I antigens expressed on the plasma membranes of W1 cells and human fetal cardiac myocytes was found to be similar (Fig. 6 B and C, H and I). G-INF was

shown to induce the neo-expression of MHC class II antigens on the plasma membranes of both W1 cells and human fetal cardiac myocytes in vitro (Fig. 6 F and L). Similarly, G-INF has been shown to induce MHC antigen expression on a variety of other cell lines in vitro (4). In addition, infusion of G-INF into rats has recently been shown to induce MHC expression on cardiac tissue in vivo (13). Thus, this cell line will provide a unique opportunity to examine MHC class I restricted cytotoxic effector cell function and, in addition, after infection with cardiotropic viruses or following sensitization, will also allow an examination of MHC class II restricted cell-mediated reactivity.

Most nucleated cells in the vertebrate species constitutively express MHC class I antigens. The W-1 cell line is no exception. Low but discrete levels of MHC class I antigens were expressed by both the W-1 cell line and the highly enriched human fetal cardiac myocytes. Of interest was the observation that the mean density of MHC class I antigens (expressed as mean channel number) expressed by the W-1 cell line was similar to that expressed by the human fetal cardiac myocytes (Fig. 6 B and C, H and I). Furthermore, in vitro incubation of the W-1 cell line and the human fetal cardiac myocytes with G-INF led not only to increased levels of MHC class I antigens, but also to neo-expression of MHC class II antigens (Fig. 6 F and L). Because G-INF has been reported to have pleiotropic effects (18), it has been argued that an in vitro treatment of cells with G-INF may result in changes in the orientation of membrane proteins, which may result in the exposure of determinants of MHC molecules in a form that is more readily accessible to reactivity with monoclonal antibodies against these determinants. To further define the nature of the increased levels of MHC class I antigens and the neo-expression of MHC class II antigens, RNA was extracted from resting and G-INF-treated W-1 cells and highly enriched populations of human fetal cardiac myocytes. Data from Fig. 7 clearly demonstrate that the increased expression of MHC class I and the neo-expression of MHC class II antigens was not due to reorientation of cryptic membrane MHC molecules, but due to increased levels of transcription and synthesis of these MHC molecules.

General considerations about the usefulness of the W1 cell line. Although W1 cells are transformed and dedifferentiated cells, they are a clone of human fetal cardiac myocytes which can be propagated indefinitely; in this respect they differ from native fetal cardiac myocytes which do not grow indefinitely. Because W1 cells do display so many phenotypic characteristics typical for human fetal cardiac myocytes, they can be used as a surrogate system to study cellular, subcellular, and molecular mechanisms, and also autoimmune mechanisms which may be implicated in myocarditis or cardiomyopathies. This surrogate system may also be useful to study the mechanisms that lead to myocyte damage and necrosis after rejection episodes after cardiac transplantation.

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